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14. ABSTRACT

Our previous studies indicated that Ii- vaccines are more efficient in CD4+ T-cell activation than Ii+ cells, and that mouse MHC II vaccines caused regression of established tumors in mice. In vitro studies with human MHC II vaccines demonstrated that the Ii- vaccines activated a population of CD4+ T-cells that is distinct from the population activated by Ii+ cells. This observation is consistent with our hypothesis that the absence of Ii results in the presentation of novel MHC II peptides. We have now identified peptides of four vaccines: MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA. For every vaccine cell line two affinity purifications were performed, and for every affinity purification two LC-MS/MS runs were conducted. Peptides present in both affinity purifications and with Peptide Prophet probability scores above 0.05 were further analyzed. One hundred sixteen peptides were identified for MCF10/DR7/CD80 and 228 peptides for MCF10/DR7/CD80/Ii; 52 peptides were present in both cell lines (Fig 1A). One hundred eight peptides were identified for MCF10/CIITA/CD80 and 28 peptides for MCF10/CIITA/CD80/Ii siRNA cells, with 6 peptides common to both cell lines (Fig 1B). These findings are consistent with our hypothesis that Ii+ and Ii- MHC II vaccines present distinct peptide repertoires. Seven peptides were chosen to be best binders by Artificial Neural Net MHC II peptide prediction by our bionformatician collaborator Dr. Michael O'Neill.

15. SUBJECT TERMS

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Table of Contents

	Page
Introduction……..…….…..	**2**
Body……..	**3**
Key Research Accomplishments……..	**4**
Reportable Outcomes……	**6**
Conclusion……	**8**
References…….	**11**
Appendices……	**12**

Introduction

Our goal is to create an efficient vaccine for the treatment of metastatic breast cancer and for prophylactic treatment of individuals at high-risk of developing breast cancer. Immune suppression induced by T-regs and MDSC is a major obstacle in immunotherapy [1, 2] . Tolerance to self antigens is another major impediment [3]. To overcome these problems, we are identifying novel tumor peptides to which the host has not previously been exposed and whose immunogenicity is not affected by immune suppression. We are focusing on identifying novel MHC class II-restricted peptides because $CD4^+$ T lymphocytes are essential for the optimal activation of cytotoxic $CD8^+$ T cells and for the generation of long-term immune memory, which is necessary for protection against latent metastatic disease [4, 5] . MHC II-restricted antigens are normally presented by professional antigen-presenting cells (APC), which endocytose exogenous proteins and degrade them to peptides in endosomal compartments. These peptides are bound by newly synthesized MHC II molecules which are directed to the endosomal compartments by the Invariant Chain (Ii), a chaperone that blocks the MHC II peptide binding groove so that peptides derived from *endogenously* synthesized proteins cannot prematurely occupy the peptide binding site [6]. In contrast to professional APC, our "MHC II" breast cancer vaccines do not contain Ii chain. MHC II vaccines are generated by transducing MHC I^+II^- tumor cells with CD80 costimulatory genes and MHC II HLA-DR molecules that are syngeneic to the prospective recipient. *We hypothesize that in the absence of Ii the vaccine cells are non-traditional APC and their MHC II molecules present an antigen repertoire of endogenous origin which is distinct from the MHC II peptide repertoire presented by professional Ii^+ APC.* Since breast cancer patients have not previously been exposed to these novel peptides, they will not be tolerized to them and the peptides should be highly immunogenic. In vivo experiments in mice demonstrated that Ii^- MHC II vaccines cause regression of established tumors, and human mammary carcinoma vaccines stimulated a much stronger $CD4^+$ T-cell response in vitro than Ii^+ APC [7]. We have also demonstrated that human lung cancer MHC II vaccines activate $CD4^+$ T-cells despite the presence of immune suppression [8]. Therefore, the MHC II vaccines are an ideal source of novel immunogenic MHC II-restricted peptides. Using an Ii^- MHC II breast cancer vaccine prepared by transducing MCF10 mammary carcinoma cells with the MHC II transactivator, CD80, and an siRNA for Ii, I have isolated and identified candidate peptides. I am currently identifying peptides from Ii^+ cells (MCF10/CIITA/CD80) and will compare them to Ii^- MHC II vaccine-derived peptides. I will then select peptides unique to the Ii^- MHC II vaccine and test them in vitro for their ability to activate $CD4^+$ T cells from breast cancer patients.

The following aims will use the MCF10/CIITA/CD80 vaccines and a single allele vaccine (MCF10/DR7/CD80).

Aim 1. Using mass spectrometry, identify peptides uniquely presented by Ii^- MCF10 MHC II vaccines and test the hypothesis that the absence of Ii facilitates the presentation of novel MHC II tumor peptides.

Aim 2. Determine if the peptides identified in Aim 1 activate type 1 tumor-reactive $CD4^+$ T-cells from breast cancer patients and if these peptides activate $CD4^+$ T-cells in the presence of immune suppression.

Aim 3. Determine if $CD4^+$ T-cells activated with the candidate peptides facilitate a more robust $CD8^+$ T-cell response by breast cancer patients.

Completion of these aims will provide "proof-of-principle" that the absence of Ii facilitates presentation of novel MHC II peptides, and will identify HLA-DR-restricted breast cancer peptides for therapeutic vaccines for patients with metastatic breast cancer.

Body

Specific Aim 1 – Completed

Our previous studies indicated that Ii^- vaccines are more efficient in $CD4^+$ T-cell activation than Ii^+ cells, and that mouse MHC II vaccines caused regression of established tumors in mice [5]. In vitro studies with human MHC II vaccines demonstrated that the Ii^- vaccines activated a population of $CD4^+$ T-cells that is distinct from the population activated by Ii^+ cells [7]. This observation is consistent with our hypothesis that the absence of Ii results in the presentation of novel MHC II peptides. We have now identified peptides of four vaccines: MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA. For every vaccine cell line two affinity purifications were performed, and for every affinity purification two LC-MS/MS runs were conducted. Peptides present in both affinity purifications and with Peptide Prophet probability scores above 0.05 were further analyzed. One hundred sixteen peptides were identified for MCF10/DR7/CD80 and 228 peptides for MCF10/DR7/CD80/Ii; 52 peptides were present in both cell lines (Fig 1A). One hundred eight peptides were identified for MCF10/CIITA/CD80 and 28 peptides for MCF10/CIITA/CD80/Ii siRNA cells, with 6 peptides common to both cell lines (Fig 1B). These findings are consistent with our hypothesis that Ii^+ and Ii^- MHC II vaccines present distinct peptide repertoires. Seven peptides were chosen to be best binders by Artificial Neural Net MHC II peptide prediction by Dr. Michael O'Neill.

Specific Aim 2 – in progress

Seven peptides identified in aim 1 (table 1) are already synthesized in the UMB biopolymer facility and T cell activation are assessed as previously described [7]. Briefly, peripheral blood mononuclear cells (PBMC) from HLA-$DR7^+$ healthy donors are used for $CD4^+$ T-cell activation experiments. To determine if the peptides identified in Objective 1 are physiologically relevant PBMC are primed by pulsing them with one of the seven peptides and incubated at 37^0. In parallel the same PBMC sample is primed by pulsing with Her2 p776 peptide as a positive control. Her2 p776 peptide is a well-studied DR7-restricted MHC II antigen, that is highly immunogenic and breast cancer specific [9]. After 6 days of incubation, cells are harvested, and expanded in medium containing 20 ng/ml of IL-15 (24 well plates at $1x10^6$ cells/2ml per well) for an additional 7 days. Expanded $CD4^+$ T cells are then rested without any cytokines for 24 hours. After resting $CD4^+$ T cells are boosted for 48 hours with irradiated MCF10/DR7/CD80, MCF10/CIITA/CD80, MCF10/CIITA/CD80/Ii siRNA, MCF10 parental cells or non-malignant MCF10A cells, and the resulting supernatants analyzed for IFNγ secretion by ELISA. If the candidate peptides are relevant tumor peptides, then the primed $CD4^+$ T cells will react (produce IFNγ) when boosted with the corresponding MCF10 vaccine cells, but not when boosted with Ii^+ MCF10 cells or with parental MCF10 or non-malignant MCF10A cells.

Key Research Accomplishments

Training plan

Task 1. Meet with my dissertation committee once a year to review my progress in the project. (Completed)
Task 2. Meet with my mentor weekly to discuss ongoing experiments and come up with and implement new experiments. Discuss my mass spectrometry data at least monthly with Dr. Fenselau; more frequently if I encounter technical problems. Update Dr. Disis periodically on candidate peptides for potential clinical trials. (Completed)
Task 3. Complete all bench laboratory research necessary to fulfill the objectives of the research proposal.(in progress)
Task 4. Complete coursework required by Molecular and Cell Biology (MOCB) Ph.D. program. (Completed)
Task 5. Oral examination on the background of my research area, and presentation and defense of my research proposal at a comprehensive "preliminary/qualifying" exam, as required by the MOCB Ph.D. program. (Completed)
Task 6: Present my research findings at one or two national conferences per year. I have presented my findings at five national conferences this past year (see reportable outcomes section)
Task 7. Write up experimental results in a timely fashion and submit them for publication to peer-reviewed journals. (I have one manuscript that has been accepted for publication; See reportable outcomes section).
Task 8. Collaborate with other students and investigators as necessary to complete my project. (on-going)
Task 9. Participate in discussions and present my work at weekly journal clubs, seminars, lab meetings, and meetings with outside speakers. (on-going)
Task 11. Serve as a teaching assistant for two semesters. (Completed).

Milestones and Deliverables:

1. Completion of my preliminary/qualifying exam). (November 2008)
2. Completed all coursework to fulfill the MOCB Ph.D. program course requirements.
3. Completed 2 semesters as a teaching assistant. .
4. Completed my first oral presentation at a national conference.
5. My first peer-reviewed publication was accepted by a well-respected journal.

Research Plan

Task 1. Identify peptides uniquely presented by Ii^- breast cancer vaccines and test the hypothesis that the absence of Ii facilitates the presentation of novel MHC II-restricted tumor peptides (completed). See fig. 1

Task 1a. Isolate MHC II-bound peptides from Ii^- and Ii^+ MCF10 cells (completed). See fig. 1

Task 1b. Using mass spectrometry analysis, compare the peptide repertoires produced by Ii^+ vs. Ii^- APC and identify candidate peptides using SEQUEST (completed). See fig. 2

Outcomes/Products/Deliverables. We obtained peptides of endogenous origin from Ii^- breast cancer vaccines, and peptides of exogenous origin from Ii^+ vaccines (completed).

Task 2. Determine if the peptides identified in Task 1 are efficacious for activating type 1 tumor-reactive $CD4^+$ T-cells from breast cancer patients (in progress).

Task 2a. Determine if the candidate peptides identified in Objective 1 activate $CD4^+$ T cells from healthy donors and from breast cancer patients that react with wild type MHC II^+ breast cancer cells (in progress).

Task 2b. Determine if the MHC II-restricted candidate peptides identified in Objective 1 are expressed by breast cancer cells (in progress).

Task 2c. Determine if MHC II tumor peptides obtained from Ii^- vaccine cells are different from peptides obtained from Ii^+ cells (in progress).

Task 2d. Determine if the peptides activate $CD4^+$ T-cells in the presence of immune suppression.

Outcomes/Products/Deliverables. This task will identify the subset of the peptides identified in Task 1 from Ii^- vaccine cells that efficiently activate tumor-reactive $CD4^+$ T-cells from breast cancer patients, even in the presence of immune suppression. (not as yet started)

Task 3. Determine if $CD4^+$ T-cells activated with the candidate peptides facilitate a more robust $CD8^+$ T-cell response by breast cancer patients.(not as yet started)

Outcomes/Products/Deliverables. This task will identify the subset of the peptides that efficiently activate type 1 tumor-reactive $CD4^+$ T-cells and facilitate the most robust $CD8^+$ T-cell response by breast cancer patients. These peptides are likely candidates for use in future breast cancer vaccines.

Reportable outcomes

Presentations

1. Olesya Chornoguz, Alexei Gapeev, Suzanne Ostrand-Rosenberg, "Mass Spectrometry Analysis of the MHC Class II peptide repertoire of cell-based breast cancer vaccines" 57th American Association for Mass Spectrometry (ASMS) Conference on Mass Spectrometry, Philadelphia, PA, June 1st – 4th, 2009 (poster).
2. Olesya Chornoguz[1], Lydia Grmai[1], Pratima Sinha[1], Konstantin Artemenko[2], Roman Zubarev[2], and Suzanne Ostrand-Rosenberg[1]. "Inflammation-induced Myeloid-Derived Suppressor Cells have enhanced resistance to apoptosis." Molecular and Cellular Biology of Immune Escape in Cancer. Keystone symposia 2010. Keystone, CO, February 7 – 12, 2010 (poster).
3. Olesya Chornoguz[1], Lydia Grmai[1], Pratima Sinha[1], Konstantin Artemenko[2], Roman Zubarev[2], and Suzanne Ostrand-Rosenberg[1]. "Inflammation-induced Myeloid-Derived Suppressor Cells have enhanced resistance to apoptosis." American Association for Cancer Research 101st annual meeting. Washington, DC. April 17-21, 2010 (poster).
4. Olesya Chornoguz[1]; Lydia Grmai[1]; Pratima Sinha[1]; Konstantin Artemenko[3]; Roman Zubarev[2]; Suzanne Ostrand-Rosenberg[1]. "Inflammation-induced Myeloid-Derived Suppressor Cells have enhanced resistance to apoptosis." American Associaiton of Immunolgists 97th Annual meeting, Baltimore, MD. May 7-11, 2010 (invited oral presentation).
5. Olesya Chornoguz[1]; Lydia Grmai[1]; Pratima Sinha[1]; Konstantin Artemenko[3]; Roman Zubarev[2]; Suzanne Ostrand-Rosenberg[1]. "Pathway Analysis reveals apoptosis as a regulator of breast cancer induced Myeloid-Derived Suppressor Cells." 58th ASMS Conference on Mass Spectrometry, Salt Lake City, UT, May 23 – 27, 2010 (oral presentation).
6. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Suzanne Ostrand-Rosenberg. "Invariant chain regulates the major histocompatibility complex class II peptide repertoire of cell-based cancer vaccines." 58th ASMS Conference on Mass Spectrometry. 58th ASMS Conference on Mass Spectrometry, Salt Lake City, UT, May 23 – 27, 2010 (poster).
7. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Michael O'Neill, Suzanne Ostrand-Rosenberg "MHC II-restricted peptides derived from invariant chain negative breast cancer cell-based vaccines activate healthy donor T-cells". AACR Tumor Immunology: Basic and clinical advances. Miami, FL, November 30 – December 3, 2010 (poster).
8. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Michael O'Neill, Suzanne Ostrand-Rosenberg "MHC II-restricted peptides derived from invariant chain negative breast cancer cell-based vaccines activate healthy donor T cells" American Associaiton of Immunolgists 98th Annual meeting. San-Francisco, CA. May 13-17, 2011, abstract submitted.

Publications

1. Olesya Chornoguz, Lydia Grmai, Pratima Sinha, Konstantin Artemenko, Roman Zubarev, and Suzanne Ostrand-Rosenberg. "Proteomic Pathway Analysis Reveals Inflammation Increases Myeloid-Derived Suppressor Cell Resistance to Apoptosis." Accepted for publication in Molecular and Cellular Proteomics journal on December 29th, 2010.

Awards

- American Association of Immunologists abstract Trainee travel Award for attendance of American Associaiton of Immunolgists 97th Annual meeting, Baltimore, MD. May 7-11, 2010 ($500), May 2010
- Washington Baltimore Mass Spectrometry Discussion Group travel award for attendance of ASMS conference in Salt-Lake City ($400), June 2010

Informatics

We are in the process of publishing the MHC II peptide data that was collected in Aim 1. As a result of this published work there will be a published database of breast cancer cells-derived MHC II peptide repertoire both in the absence and the presence of Invariant Chain. This database will facilitate development of future breast cancer peptide vaccines.

Conclusions

- Task 1 of Research plan was successfully completed (outlined in Statement of Work, SOW). MHC II peptides presented by Ii^+ and Ii^- MHC II breast cancer vaccines were identified.
- It has been demonstrated that Ii^+ and Ii^- vaccines present distinct, but overlapping peptide repertoire
- It has also been showed that Invariant chain influences MHC II peptide repertoire
- Seven peptides predicted to have the highest affinity to MHC II molecules were chosen for in vitro $CD4^+$ T-cell activation and are currently being tested.
- Most relevant specific tasks outlined in Training Plan and Milestones and Deliverables sections of SOW were completed.
- More specifically I have presented several posters and gave two oral presentations at several national meetings during the first year of training period.
- I have also received two travel awards to present my research at AAI and ASMS conferences (see reportable outcomes section).
- My first first-author manuscript titled "Proteomic Pathway Analysis Reveals Inflammation Increases Myeloid-Derived Suppressor Cell Resistance to Apoptosis" has been submitted and accepted for publication in Molecular and Cellular Proteomics journal.

References

1. Ostrand-Rosenberg, S., *Immune surveillance: a balance between protumor and antitumor immunity.* Curr Opin Genet Dev, 2008. 18(1): p. 11-8.
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4. Janssen, E.M., et al., *CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes.* Nature, 2003. 421(6925): p. 852-6.
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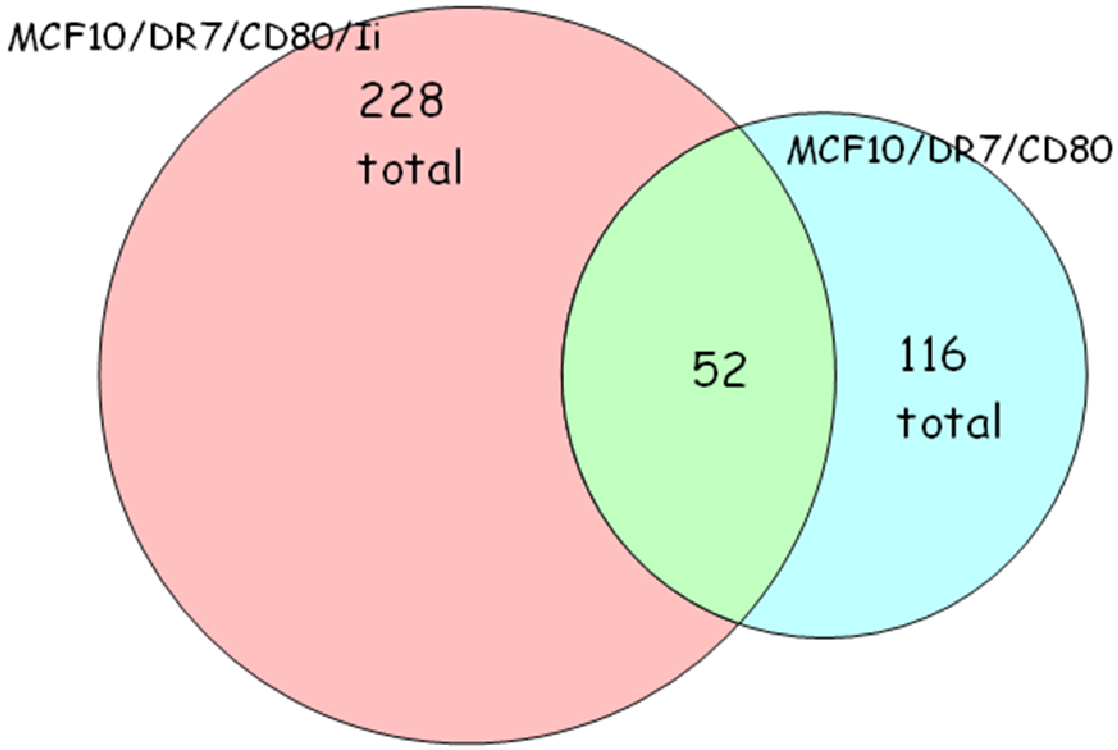
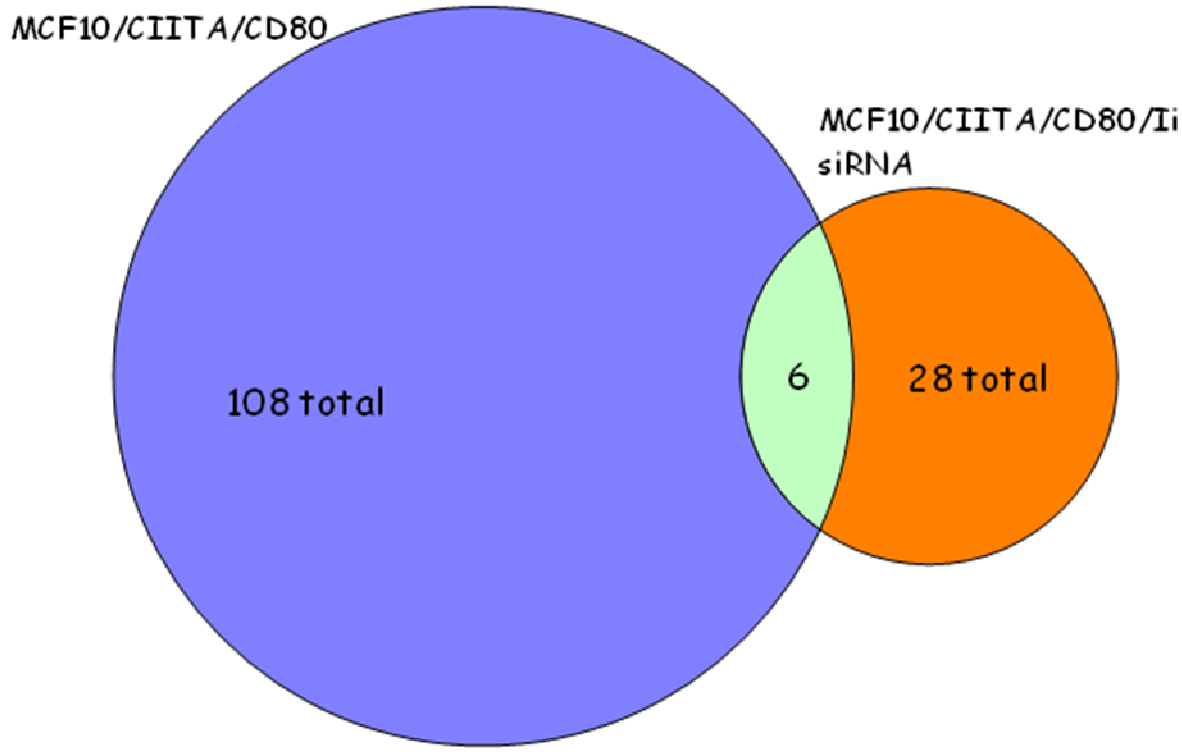
7. Thompson, J.A., et al., *The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4+ T lymphocytes.* Cancer Immunol Immunother, 2008. 57(3): p. 389-98.
8. Srivastava, M.K., et al., *Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells.* Cancer Immunol Immunother, 2008. 57(10): p. 1493-504.
9. Sotiriadou, R., et al., *Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope.* Br J Cancer, 2001. 85(10): p. 1527-34.

Fig 1. **MHC II peptides identified from 4 vaccines.** These peptides were identified as a result of 2 MHC II peptide purifications. Peptides from each purification were analyzed twice on LTQ mass spectrometer; only peptides common between the two purifications were considered as reliable identifications.

A)



B)



Sequence and SOR#	Protein	Vaccine	Sub cellular localization
RQTVAVGVIKAVDKKA, SOR-42	Translation elongation factor, EEF1A1 protein	MCF10/CIITA/CD80/ Ii siRNA, present in Ii + as well	Nucleus, nucleolus, cytoplasm
KVAPAPAVVKKQEAKK, SOR-43	60S ribosomal protein L7a	MCF10/CIITA/CD80/ Ii siRNA	Predicted: Mitochondrion and cytoplasm
EEEALANASDAELCDIAAILGMHTLM, SOR-44	Tropomodulin 1	MCF10/DR7/CD80, present in Ii+ as well	Predicted: Nucleus and cytoplasm
SESAAAPAFASSSSEVNPAPKFHW, SOR-46	Hypothetical protein, isoform 1	Both	Protein not present in the database
RTYAGGTASATKVSASSGATSKS, SOR-45	Calpastatin, isoform e, calcium-dependent cysteine protease) inhibitor	Both	Protein not present in the database
GKFYLVIEELSQLFRSLVPIQL, SOR-48	Hypothetical protein	MCF10/CIITA/CD80/ Ii siRNA	Protein not present in the database
RRMRLTHCGLQEKHL, SOR-47	NLRC5 protein, IFN-specific response element	MCF10/DR7/CD80	Predicted: Cytoplasm, nucleus

Table 1. Seven peptides that were derived from Ii$^-$ vaccines and were predicted to have the highest binding affinity to HLA-DR7 MHC II, using Artificial Neural Networks (ANN). The first column shows peptide sequence and a short-hand notation (SOR-xx number). Second column shows the protein from which the peptide was derived. Some of the peptide (SOR-46 and SOR-48) are derived from peptides of unknown function, thus they are named as hypothetical proteins in the FASTA database. The third column shows which vaccine or vaccines the peptide was derived from. The fourth column shows predicted or published sub cellular localization of the proteins from which the peptides were derived from. LOCATE web-tool

(http://locate.imb.uq.edu.au) was used to derive predicted or published sub cellular localization data. Three of the proteins of interest were not present in LOCATE database, and therefore are indicated as “Protein not present in the database”. Peptides highlighted in blue have already been tested for their ability to activate $CD4^+$ T-cells as measured by IFNγ secretion

Proteomic Pathway Analysis Reveals Inflammation Increases Myeloid-Derived Suppressor Cell Resistance to Apoptosis.

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Running Title: Inflammation increases MDSC resistance to apoptosis

ABBREVIATIONS

DAPI	4’,6-diamidino-2-phenylindole
FAM	carboxyfluorescein
FasL	Fas ligand (CD95L)
IL-1β	interleukin-1β
IL-6	interleukin-6
IPI	International Protein Index
MAPK	mitogen activated protein kinase pathway
MCF	mean channel fluorescence
MDSC	myeloid-derived suppressor cells
mAb	monoclonal antibody
MOWSE	molecular weight search
TCCM	tumor cell conditioned medium
TGF-β	transforming growth factor beta
TLR4	Toll-like receptor 4
ND	Not detected

SUMMARY

Myeloid-derived suppressor cells (MDSC) accumulate in patients and animals with cancer where they mediate systemic immune suppression and obstruct immune-based cancer therapies. We have previously demonstrated that inflammation, which frequently accompanies tumor onset and progression, increases the rate of accumulation and the suppressive potency of MDSC. To determine how inflammation enhances MDSC levels and activity we used mass spectrometry to identify proteins produced by MDSC induced in highly inflammatory settings. Proteomic pathway analysis identified the Fas pathway and caspase network proteins, leading us to hypothesize that inflammation enhances MDSC accumulation by increasing MDSC resistance to Fas-mediated apoptosis. The MS findings were validated and extended by biological studies. Using activated caspase 3 and caspase 8 as indicators of apoptosis, flow cytometry, confocal microscopy, and western blot analyses demonstrated that inflammation-induced MDSC treated with a Fas agonist contain lower levels of activated caspases, suggesting that inflammation enhances resistance to Fas-mediated apoptosis. Resistance to Fas-mediated apoptosis was confirmed by viability studies of MDSC treated with a Fas agonist. These results suggest that an inflammatory environment, which is frequently present in tumor-bearing individuals, protects MDSC against extrinsic-induced apoptosis resulting in MDSC with a longer in vivo half-life, and may explain why MDSC accumulate more rapidly and to higher levels in inflammatory settings.

INTRODUCTION

Immunotherapies aimed at activating the host's immune system are promising strategies for the treatment of cancer because of their potential for minimal toxicity to healthy cells and their ability to induce immune memory that may protect against metastatic disease (1). Disappointingly, clinical trials of most cancer vaccines or other active T-cell mediated immunotherapies have not yielded significant patient responses (2). Since most cancer patients are immune suppressed, these failures are most likely due to the inability of cancer patients to immunologically respond to the immunotherapy agents. Although multiple mechanisms contribute to immune suppression in individuals with cancer (3), myeloid-derived suppressor cells (MDSC) accumulate in virtually all cancer patients and are a major cause of tumor-induced immune suppression due to their inhibition of both adaptive and innate immunity (4). Because of their widespread presence and potent immune suppressive effects, identifying the cellular and molecular mechanisms responsible for MDSC accumulation and suppressive activity may facilitate the development of effective immunotherapy strategies.

Chronic inflammation frequently precedes tumor onset (5) and many cancer cells produce pro-inflammatory mediators, suggesting that chronic inflammation contributes to tumorigenesis and tumor progression (6). We and others have previously demonstrated that inflammation via the pro-inflammatory molecules Interleukin (IL)-1β (7,8), Toll-like receptor 4 (TLR4) (9), IL-6 (10), prostaglandin E2 (11,12), and S100A8/A9 proteins (13,14) increases either the number or the suppressive potency of MDSC, or both. This causative relationship between inflammation and MDSC induction led us to hypothesize that MDSC not only are an obstacle to immunotherapy, but also

contribute to the onset and progression of tumors by inhibiting immune surveillance of newly transformed cells and by blocking natural immunity to established tumors (15).

We are studying the effects of inflammation on tumor progression and MDSC development using the mouse BALB/c-derived, spontaneously metastatic 4T1mammary carcinoma (16) transfected with the IL-1β gene (4T1/IL-1β) (7). When inoculated into the mammary fat pad of syngeneic BALB/c mice, wild type 4T1 and 4T1/IL-1β tumor cells form a primary tumor at the site of injection and spontaneously metastasize to the lungs, liver, brain, lymph nodes (16), and bone marrow (17). Increasing tumor burden drives the accumulation of $Gr1^{+}CD11b^{+}$ MDSC in bone marrow, spleen, blood, and at the site of primary and metastatic tumor (13). Heightened inflammation in the form of high levels of IL-1β in the tumor microenvironment exacerbates tumor progression through various mechanisms. For example mice with 4T1/IL-1β tumors have a shorter survival time, and contain more MDSC that are more suppressive as compared to mice with 4T1 tumors (7,8). IL-1β also increases the ability of MDSC to induce tumor-promoting macrophages through a Toll-like receptor 4 (TLR4)-dependent mechanism (9), and increases the in vivo half-life of MDSC (7,8).

Pathways and proteins that differ between MDSC induced in highly inflammatory ("inflammatory" MDSC) vs. less inflammatory ("conventional" MDSC) environments may be potential drug targets for eliminating or reducing MDSC activity. Therefore, we have used mass spectrometry based quantitative proteomic analysis followed by pathway analysis to identify activated pathways and proteins of inflammatory MDSC induced by 4T1/IL-1β tumor vs. conventional MDSC induced by 4T1 tumor. Because TLR4 transmits signals that increase MDSC potency, we have also compared the protein and

pathway profiles of MDSC induced in wild type BALB/c mice vs. TLR4-deficient mice. Proteomic analysis revealed numerous regulated proteins, while pathway analysis identified several pathways that were up-regulated in inflammatory vs. conventional MDSC, and in wild type vs. TLR4-deficient mice. Interestingly, the Fas pathway and caspase network were up-regulated in inflammatory MDSC from BALB/c mice, and the caspase network was up-regulated in conventional MDSC from $TLR4^{-/-}$ mice. Since caspase network proteins are the effector molecules of Fas-mediated apoptosis, we hypothesized that MDSC survival and half-life in vivo may be regulated by apoptosis. We have confirmed this hypothesis and demonstrate that inflammatory MDSC have enhanced resistance to Fas-mediated apoptosis. These results suggest that inflammation protects MDSC against extrinsic-induced apoptosis resulting in MDSC with a longer in vivo half-life, and may explain why MDSC accumulate more rapidly and to higher levels in inflammatory tumor environments.

EXPERIMENTAL PROCEDURES

Mice and cell lines - TLR4$^{-/-}$, TS1 (T-cell receptor transgenic for influenza hemagglutinin peptide 110-115 restricted to I-E^{d}; (18)), DO11.10 (T cell receptor transgenic for ovalbumin peptide 323-339 restricted to I-A^{d}; (19)), and wild type BALB/c mice were obtained from The Jackson Laboratory and bred and maintained in the University of Maryland Baltimore County animal facility. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee. 4T1 and 4T1/IL-1β cell lines were maintained as described (7).

Tumor inoculations and MDSC harvesting - Female 6- to 10-wk-old BALB/c and TLR4$^{-/-}$ mice were inoculated in the abdominal mammary fat pad with 1x10^{5} 4T1 or 4T1/IL-1β cells. Approximately three weeks later when tumors were 9 to 11 mm in diameter and metastatic disease was established, mice were bled from the submandibular vein. Red blood cells were removed by lysis. Samples in which 90% or more of the remaining leukocytes were Gr1^{+}CD11b^{+} MDSC as measured by flow cytometry were used.

T-cell activation - CD4^{+} T-cell activation was done as described (20). Briefly, TS1 or DO 11.10 splenocytes were co-cultured with their cognate peptide and irradiated blood MDSC, pulsed on day four with ^{3}H-thymidine, and harvested 24 hours later. Samples were run in triplicate and were counted in a scintillation counter. Data are expressed as the percent suppression relative to transgenic splenocytes plus peptide without MDSC. Percent suppression = 100% x ([(cpm splenocytes + peptide) – (cpm splenocytes + peptide + MDSC)]/(cpm splenocytes + peptide)).

Reagents and antibodies - All chemicals were purchased from Sigma unless noted otherwise. Fluorescently coupled monoclonal antibodies (mAbs) to Gr1, CD11b, Fas (CD95), activated caspase-3, and isotype control mAbs were obtained from BD Pharmingen. 4',6-diamidino-2-phenylindole (DAPI) and caspase-8 carboxyfluorescein (FAM) reagent were purchased from Invitrogen. Activated caspase-3 polyclonal antibody for Western blot was purchased from Abcam.

Apoptosis assay - 4T1 tumor cells were grown to confluency in 20 ml of culture medium in T-75 flasks and the supernatants were collected and used as tumor cell conditioned media (TCCM). MDSC were cultured in 1:1 TCCM:culture medium for 12-20 hr with Jo-2 (Fas agonist; BD Pharmingen) or isotype control (1µg/ml) mAb.

Flow cytometry and confocal microscopy - Intracellular and cell surface fluorescence staining for flow cytometry was performed as described (20). $Gr1^+CD11b^+$ cells were gated and analyzed for expression of caspase 3, caspase 8, or Fas using a Cyan ADP flow cytometer and Summit analysis software (Beckman/Coulter). Percent positive cells was determined by comparing the staining with specific mAb vs. isotype control mAb. Numerical values shown are the mean channel fluorescence (MCF) for the indicated, gated populations. For microscopy, live MDSC were adhered to cover slips coated with Cell Tak (BD Pharmingen) for 20 min at room temperature, washed twice with excess PBS, and fixed for 10 min at 37^{o}C with 2% formaldehyde in PBS. Fixed cells were washed with excess PBS containing 10% FCS (PBS-10% FCS) for 20 minutes. For cell surface markers, cells were then stained in the dark for 1hr at 4^{o}C with antibodies diluted in PBS-10% FCS, followed by washing with excess PBS-10% FCS. For intracellular staining antibodies were diluted in PBS-10% FCS containing 0.2%

saponin, followed by a 10 min incubation with DAPI in PBS (5 µg/ml), and a final wash in PBS-10% FCS. Cover slips were mounted on slides with 10 µl of SlowFade (Invitrogen), visualized using a Leica TCS SP5 Broadband Tandem scanning confocal microscope fitted with an HCX PL APO 63x 1.4 numerical aperture objective, and analyzed using Leica Image Browser software. Fluorescent images were collected sequentially. Approximately 16 optical slices through the z-plane were collected for each field. A minimum of 20 cells in multiple fields was counted for each sample.

Western blots - Western blots were performed according to the manufacturer's (Abcam) protocol except blocking was performed overnight. Bands were quantified using ImageJ software and measuring the mean density of the activated caspase-3 and β-actin bands. Normalized density of activated caspase 3 = (activated caspase-3 mean density/β-actin mean density).

Sample preparation and LC-MS/MS - MDSC (>90% $Gr1^+CD11b^+$ cells; $5x10^6$ – 10^7/mouse) were lysed at a final concentration of 0.1% Rapigest acid-cleavable detergent (Waters) in 100 mM NH_4HCO_3, pH 8.4. Cell lysates were digested with sequencing grade modified trypsin (1:50 trypsin:protein ratio; Promega; cleaves after arginine or lysine) for 1 hr at 37^0C, after which trifluoroacetic acid was added to a final pH of ~3. The resulting material was incubated for 1 hr at 37^0C, then freeze-thawed at -80^0C to ensure detergent precipitation, and microfuged at 13,200 rpm for 5 minutes (Eppendorf 5415 D microfuge).The acid-precipitated material was then discarded (21). Supernatant containing tryptic peptides was collected, brought to pH 7, and peptide concentration was measured by OD 280. Thirty µg of peptides were desalted using C18 spin cartridges (Pierce), and analyzed using an LTQ-FT Ultra mass spectrometer

(ThermoFischer) interfaced with an Agilent 1100 nanoLC system. A 15-cm fused silica emitter (75-µm inner diameter, 375-µm outer diameter; Proxeon Biosystems) was used as a nanocolumn. The emitter was packed in-house with methanol slurry of reversed-phase, fully end-capped Reprosil-Pur C18-AQ 3 µm resin (Maisch GmbH) using a pressurized “packing bomb” operated at 50-60 bars (Proxeon Biosystems). The sample aliquots were injected into the nanocolumn and peptide elution was performed at a flow rate of 200 nL/min with mobile phases A (water with 0.5% acetic acid) and B (89.5% acetonitrile, 10% water, and 0.5% acetic acid). A 100-min gradient from 2% B to 50% B followed by a washing step with 98% B for 5 min was used. Mass spectrometric analysis was performed using unattended data-dependent acquisition mode, in which the mass spectrometer automatically switches between acquiring a high resolution survey mass spectrum in the FT detector (resolving power 100,000) and consecutive low-resolution, collision-induced dissociation of up to five most abundant ions in the linear ion trap. MDSC samples from an individual mouse were analyzed twice by LC-MS/MS. One biological sample per group was analyzed by Pathway Search Engine.

Database searching and criteria - Acquired data (RAW-files) were converted to the mgf format using an in-house program (C++) and submitted to the database search engine Mascot (version 2.1.3, Matrix Science). Database search (NCBI database 20070109; 4396331 sequences; 1512170332 residues) was performed with the following conditions: trypsin specificity, peptide mass tolerance set to 10 ppm; fragment mass tolerance at 1.5 Da; a maximum of two allowed missed cleavages; carbamidomethyl, deamidation, methionine oxidation and disulfide as the variable modifications. Proteins that were identified by two or more peptides with a Mascot

MOWSE score above 30 were considered as reliable identifications. Protein identification probability was derived from Mascot Mowse score (22). Mascot output peptide list in the html-format and the corresponding mgf and RAW files of each sample were used as input files for quantification using an in-house program package (C++). The identified unique ('bold red' in Mascot) peptides were then searched in each LC-MS/MS dataset using their accurately known molecular mass and approximately known retention time.

Label-free quantification - Quantification of the peptide expression levels was based on the chromatographic peak areas and was carried out using an in-house program. The area of the chromatographic peak was considered the peptide abundance. Sum of the abundances of all 'bold red' non-identical peptides was attributed to the protein expression level (≥2 peptides per protein). Normalization was done as previously described (23)

Proteomic pathway analysis - Pathway analysis was done as described (24). Briefly, lists of protein identifiers, namely International Protein Index (IPI) entry names together with their relative abundance values and the highest Mascot score the protein received were loaded into ExPlain^{TM} 1.3 tool (BIOBASE). Upstream key-nodes most relevant for the input gene products were then identified. Key-nodes (also known as bottle-necks) are signalling molecules found on pathway intersections in the upstream vicinity of the genes from the input list. Each key-node was given a score reflecting its connectivity, i.e. how many input-list genes were reached and the proximities to those genes. The score calculation also included the abundances of the downstream proteins

detected in the proteomics experiment. Changes in the key node scores thus reflected the changes in the activation levels of the corresponding signalling networks.

Statistical analysis – Percent suppression (figure 1C) and flow cytometry for caspase 3 expression (figure 4) were analyzed by the SIGN test. Values for percent dead cells (fig. 4D) and for suppressive activity of MDSC (fig. 1C) were analyzed by Student's *t* -test. MS pathway analysis and statistical scoring were done using Pathway Search Engine (24).

RESULTS

MDSC Accumulate in Tumor-bearing Mice and Suppress T-cell Activation - BALB/c and TLR4$^{-/-}$ mice were inoculated in the mammary fat pad with 4T1 or 4T1/IL-1β cells on day 0 and bled on day 21-24 when primary and metastatic tumors were established (**Fig. 1A**). Red blood cells were removed by lysis, and the remaining leukocytes were assayed by flow cytometry to ascertain their identity as MDSC. Greater than 92% of the remaining leukocytes in all groups had the Gr1^{+}CD11b^{+} phenotype characteristic of MDSC (**Fig. 1B**). In agreement with previous studies and indicative of the heterogeneity of MDSC, Gr1 phenotypes ranged from Gr1^{med} to Gr1^{hi} (7,9,10), with inflammation-induced (IL-1β-induced) MDSC displaying the greatest heterogeneity (25). To ensure that the Gr1^{+}CD11b^{+} cells were functional MDSC, they were co-cultured at varying ratios with naïve splenocytes from transgenic mice in the presence of cognate peptide (**Fig. 1C**). All populations of Gr1^{+}CD11b^{+} cells suppressed T cell activation, with 4T1-induced MDSC from BALB/c mice being the least suppressive. Therefore, the cell populations used in subsequent experiments consist of >92% immune suppressive MDSC.

Proteomic and Pathway Analysis of Inflammation-induced Proteins in MDSC - To elucidate the effects of inflammation on MDSC we compared conventional MDSC (4T1-induced MDSC from BALB/c mice) vs. inflammatory MDSC (4T1/IL-1β-induced MDSC from BALB/c mice). Because of its role in enhancing MDSC-macrophage cross-talk, we also compared MDSC capable of signaling through the TLR4 receptor (4T1-induced MDSC from BALB/c mice) vs. MDSC unable to signal through TLR4 (4T1-induced MDSC from TLR4$^{-/-}$ mice). MDSC characterized in fig. 1 were prepared for LC-MS/MS

analysis using Rapigest and digested by trypsin, and peptide samples were analyzed using a LTQ-FT Ultra mass spectrometer (**Fig. 2**). Seven hundred and eighty-nine proteins were present in both 4T1 and 4T1/IL-1β BALB/c MDSC, and were identified and quantified using a label free approach. 4T1-induced MDSC from TLR4$^{-/-}$ and BALB/c mice shared 749 proteins which were similarly identified and quantified. Supplemental tables 1 and 2 show the NCBI accession number, protein name, normalized relative abundance, and derived protein identification probability. for these proteins. Resulting protein identifications and relative protein abundances were loaded into ExPlain, converted into gene identifications, and the gene identifications were then mapped to upstream key nodes in cellular pathways. Each key-node was given a score reflecting its connectivity, i.e. how many input-list genes were reached and the proximities to those genes, as well as abundances of the downstream proteins measured by LC/MS. Changes in the key node scores thus reflected the changes in the activation levels of the corresponding signalling networks. The pathway score was represented by the sum of the scores of all key node that are part of that pathway.

Two pathway analyses comparisons were conducted: 4T1-induced MDSC from BALB/c mice (control) vs. 4T1/IL-1β-induced MDSC from BALB/c mice (experimental); and 4T1-induced MDSC from BALB/c mice (control) vs. 4T1-induced MDSC from TLR4$^{-/-}$ mice (experimental). Fourteen significantly up-regulated pathways and 9 significantly down-regulated pathways were detected in 4T1/IL-1β induced MDSC from BALB/c mice (**Fig. 3A**). Predominant pathways included the caspase network, Fas, TGF-β, and IL-1 pathways. Among the most highly up-regulated pathways were the caspase network

and Fas pathway. For 4T1 TLR4$^{-/-}$ MDSC there were 5 significantly up-regulated pathways and 2 significantly down-regulated pathways (**Fig. 3B**).

The Fas pathway is the extrinsic apoptosis pathway and is activated when Fas ligand (FasL; CD95L) on an effector cell binds to Fas on the plasma membrane of the target cell. These interactions result in the cleavage of caspase 8 and the subsequent cleavage and activation of caspase 3, which in turn leads to cell apoptosis (26). Multiple proteins associated with the Fas pathway were identified by MS in all MDSC samples, including caspase-3, caspase-8, lamin B1 and Rho GDP inhibitor beta (Rho GDI Beta) (**Fig. 3C**). The convergent identification of Fas pathway-associated proteins in MDSC samples suggests that MDSC accumulation, maintenance, and survival in vivo may be regulated by apoptosis. This hypothesis provides a mechanistic explanation for our previous reports that inflammation increases the rate of accumulation of MDSC in tumor-bearing mice (7,10), and led us to validate the MS findings with biological experiments.

Inflammation-induced MDSC Are More Resistant to Fas Mediated Apoptosis – To determine whether inflammation increases MDSC by reducing their susceptibility to Fas-mediated apoptosis, we assessed the expression of activated caspase-3 and caspase-8 in conventional vs. inflammatory MDSC. If inflammation increases MDSC levels by reducing their apoptotic rate, then treatment of inflammatory MDSC with a Fas agonist will yield lower levels of activated caspase 3 and caspase 8 relative to treatment of conventional MDSC. To test this possibility, BALB/c mice were inoculated with 4T1/IL-1β or 4T1 cells and blood MDSC were collected 21-24 days later. MDSC were incubated overnight with or without the Fas agonist Jo2 mAb, labeled for Gr1, CD11b,

and activated caspase 3 or caspase 8, and the gated $Gr1^+CD11b^+$ cells analyzed by flow cytometry. 4T1/IL-1β-induced BALB/c MDSC have consistently less activated caspase-3 as compared to 4T1-induced MDSC (percent caspase 3^+ cells: 58.6% vs. 65.6%; mean channel fluorescence (MCF) of caspase 3^+ cells: 76.9 vs. 87.9, respectively) (**Fig. 4A**). Caspase 8 expression was similarly reduced in inflammatory vs. conventional MDSC following Jo2 treatment (61.6% vs. 64.9% caspase 8^+ cells; 160.5 vs. 191.4 MCF of caspase 8^+ cells, respectively) (**Fig. 4B**). These data indicate that inflammatory MDSC are less susceptible to Fas-mediated apoptosis as compared to conventional MDSC.

To confirm the flow cytometry findings, lysates of conventional (4T1-induced) and inflammatory (4T1/IL-1β-induced) MDSC were electrophoresced by SDS-PAGE, western blotted with mAbs to activated caspase 3, and the bands quantified by densitometry (**Fig. 4C**). Jo2-treated conventional MDSC contain 1.75 fold more activated caspase 3 relative to inflammatory MDSC, confirming that inflammatory MDSC contain less activated caspase 3 and are more resistant to Fas-mediated apoptosis vs. conventional MDSC.

The decreased susceptibility of inflammatory MDSC could be due to differences in the percent of MDSC that express Fas, or to lower levels of plasma membrane Fas on inflammatory vs. conventional MDSC. To test this possibility, 4T1/IL-1β-induced and 4T1-induced MDSC were stained with fluorescent mAbs to Gr1, CD11b, and Fas, and the gated $Gr1^+CD11b^+$ cells were analyzed by flow cytometry for Fas expression (**Fig. 4D**). Conventional and inflammatory MDSC populations contain similar percentages of Fas^+ cells (85.7% and 84.1%, respectively), and conventional MDSC have slightly less

Fas relative to inflammatory MDSC (MCF of 19.2 and 26.0, respectively). Therefore, the reduced levels of activated caspase 3 and caspase 8 in inflammatory MDSC are not due to less plasma membrane Fas on inflammation-induced cells.

To ascertain that the lower levels of activated caspase 3 and caspase 8 in inflammatory MDSC result in higher survival, we measured MDSC viability following treatment with the Fas agonist Jo2 mAb (**Fig. 4E**). There is background cell death in both 4T1/IL-1β-induced and 4T1-induced MDSC; however, a significantly higher percentage of 4T1-IL-1β-induced MDSC are viable following Fas agonist treatment, confirming that inflammatory MDSC are less susceptible to Fas-mediated apoptosis.

To further demonstrate that inflammatory MDSC are less susceptible to Fas-mediated apoptosis, 4T1/IL-1β and 4T1-induced MDSC from BALB/c mice were analyzed by confocal microscopy. The two populations of MDSC were treated with the Fas agonist Jo2 mAb or an irrelevant control mAb, and stained with DAPI and fluorescent mAbs to Gr1, CD11b, and activated caspase 3 (**Fig. 5**). MDSC were identified by their characteristic multi-lobed nuclei as revealed by DAPI nuclear staining and their co-expression of Gr1 and CD11b. Ninety-five (20/21 cells) and 100% (25/25 cells) of control mAb-treated conventional and inflammatory MDSC, respectively, were dimly stained with the caspase 3 mAb, indicative of low levels of activated caspase 3 in the absence of Fas signaling. In contrast, 100% (16/16 cells) of Jo2-treated conventional MDSC were brightly stained, while none (0/37) of the Jo2-treated inflammatory MDSC were brightly stained, further demonstrating that inflammatory MDSC are more resistant to Fas-mediated apoptosis. These results agree with the MS,

flow cytometry and western findings, and further confirm that inflammation limits the activation of caspase 3 and protects MDSC against Fas-mediated apoptosis.

DISCUSSION

The association of chronic inflammation and tumor onset and progression was first observed more than 100 years ago (27). However, it is only recently that the mechanisms linking inflammation and cancer, and particularly the role of the immune system, are being clarified (28). An individual's immune system has the capacity to reduce or eliminate malignant cells; however, tumors frequently evade anti-tumor immunity. Paradoxically, many of the evasive mechanisms involve co-opting the function of immune cells(3). MDSC are a major contributor to tumor immune escape due to their potent suppression of innate and adaptive anti-tumor immunity and their widespread presence in most cancer patients (29). In healthy, tumor-free mice $Gr1^+CD11b^+$ cells represent <~10% of leukocytes in the blood, while mice with large mammary tumors can have up to 95% of their circulating leukocytes as $Gr1^+CD11b^+$ cells (15). Because these profoundly immune suppressive $Gr1^+CD11b^+$ MDSC are present at such high levels and because inflammation increases MDSC levels and suppressive activity, we are studying how inflammation drives MDSC accumulation and function with the goal of identifying proteins and/or signaling pathways that could serve as drug targets for reducing MDSC-mediated immune suppression. In this first proteomic analysis of MDSC, we identified 709 proteins synthesized by conventional (4T1-induced) and inflammatory (4T1/IL-1β-induced) MDSC from BALB/c mice and 675 proteins from 4T1-induced MDSC from $TLR4^{-/-}$ mice. Numerous pathways were up-regulated in all MDSC populations. Of particular interest was up-regulation of the Fas pathway and caspase network in inflammatory MDSC from BALB/c mice because of their potential role in MDSC turnover. Based on the MS analysis, we hypothesized that

inflammation promotes MDSC survival by regulating Fas-mediated apoptosis of MDSC. Flow cytometry, western analyses, confocal microscopy, and cell viability studies following treatment of conventional MDSC vs. inflammatory MDSC with a Fas agonist confirmed our hypothesis and demonstrated that inflammation increases MDSC accumulation and potency by generating MDSC that are less susceptible to Fas-mediated apoptosis. These studies solidify the causative relationship between inflammation, tumor progression, and immunity, and provide a mechanistic explanation for how inflammation facilitates malignancy via the immune system.

In addition to the caspase and Fas pathways, other pathways were also up-regulated in inflammation-induced vs. conventional MDSC, and these pathways are also likely to regulate MDSC maintenance as well as function. For example, TGFβ is a suppressive cytokine that is produced by other suppressive cells as well as by MDSC (30) and its increased expression in inflammation-induced MDSC could contribute to heightened suppressive activity. Likewise, stress is associated with the induction of MDSC (31), and increased expression of the stress pathway is therefore likely to regulate MDSC levels. Interestingly, the caspase network is also up-regulated in 4T1-induced MDSC from BALB/c vs. $TLR4^{-/-}$ mice, suggesting that MDSC generated in $TLR4^{-/-}$ mice may turn-over more quickly than MDSC in wild type BALB/c mice. Additional biological studies are needed to determine if the TGFβ and stress pathways significantly facilitate the survival of inflammation-induced MDSC and whether the half life of MDSC from $TLR4^{-/-}$ mice is controlled by proteins of the caspase pathway.

The lower number of activated pathways observed in MDSC from $TLR4^{-/-}$ vs. $TLR4^{+/+}$ mice is not unexpected. In addition to their suppressive effects on T

lymphocytes, MDSC also facilitate tumor growth by polarizing macrophages towards a tumor-promoting type 2 phenotype (32). Previous studies established that MDSC from TLR4$^{-/-}$ mice are less able to induce macrophage polarization (9). This characteristic is consistent with the observation that fewer pathways are activated in TLR4$^{-/-}$ MDSC, and suggests that the pathways that are absent in TLR4$^{-/-}$ MDSC may be responsible for MDSC-mediated macrophage polarization. Activation of the caspase network in TLR4$^{-/-}$ MDSC is also not unreasonable and indicates that MDSC turnover is regulated independently of how MDSC are activated.

There are several potential mechanisms by which inflammation could increase MDSC resistance to apoptosis. Fas-mediated apoptosis is inhibited by the intracellular protein c-FLIP which prevents the activation of caspase 8 (33). However, c-FLIP was not over-expressed in our proteomic screen and there is no difference in c-FLIP levels between conventional and inflammatory MDSC treated with the Fas agonist (data not shown), so c-FLIP does not regulate resistance of 4T1/IL-1β-induced MDSC. Fas-mediated apoptosis is also regulated by the Mitogen-activated Protein Kinase (MAPK) pathway (34). When activated by phosphorylation, p38 kinase inhibits the activation (auto-cleavage) of caspase-3 and caspase-8 (35). Multiple stimuli induce p38 activation, including transforming growth factor beta (TGF-β (36)), which directly down-regulates Fas-mediated apoptosis (37). Interestingly, our pathway analysis identified both TGF-β and p38 pathways as up-regulated in inflammatory MDSC as compared to conventional MDSC (**Fig. 3A**), suggesting that elevated levels of TGF-β in inflammatory MDSC may either directly or indirectly through the MAPK pathway, protect MDSC from apoptosis.

Regardless of the mechanism by which inflammation exacerbates MDSC survival and function, our proteomic and pathway analysis and validation studies demonstrate that inflammation facilitates MDSC survival by increasing MDSC half-life. In previous studies we observed that surgical removal of primary 4T1 mammary tumors resulted in a significant decrease in circulating MDSC, while circulating MDSC levels remained elevated in mice whose 4T1/IL-1β tumors were removed (7). We have also reported that inflammation increases the rate of accumulation of MDSC in tumor-bearing mice (7,10). We proposed that both of these effects could be due to either increased production of MDSC and/or an increase in the half-life of MDSC. The current studies support the concept that these effects are the result of an increase in half life due to increased resistance to apoptosis.

Our studies not only provide a mechanistic explanation for the longevity of inflammation-induced MDSC, but they also identify proteins and pathways that are potential drug targets for perturbing MDSC viability and facilitating active immunotherapies. In addition, these studies are another example of the usefulness of quantitative pathway analysis and the new analytical tool, Pathway Search Engine for analyzing proteomics data and generating knowledge-based predictions of signal transduction pathways.

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Figure Captions

Fig 1. Cells isolated from tumor-bearing BALB/c or TLR4$^{-/-}$ mice are Gr1^{+}CD11b^{+} MDSC that suppress antigen-specific T cell activation. *A*, BALB/c or TLR4-deficient mice were inoculated on day 0 in the abdominal mammary fat pad with 10^5 4T1 or 4T1/IL-1β cells. Three weeks post inoculation primary tumors and metastatic disease were established and the mice were bled for MDSC. *B,* Red blood cells were removed from the blood samples described in panel A and the remaining leukocytes were stained with fluorescent mAbs to the MDSC markers Gr1 and CD11b and analyzed by flow cytometry. Percent of leukocytes that were Gr1^{+}CD11b^{+} are indicated. Data are from one of three independent experiments with MDSC from one mouse per group. *C*, Splenocytes from TS1 (4T1 and 4T1/IL-1β-induced MDSC from BALB/c mice) and DO11.10 (4T1-induced MDSC from TLR4$^{-/-}$ mice) transgenic mice were co-cultured in the presence or absence of cognate peptide and decreasing numbers of MDSC. T-cell activation was measured by incorporation of tritiated thymidine and plotted as percent suppression relative to the absence of MDSC. Data are from one of two independent experiments with MDSC from one mouse per group.

Fig. 2. Proteomic analysis workflow. MDSC were harvested from BALB/c mice with 4T1 tumor, BALB/c mice with 4T1/IL-1β tumor, and TLR4$^{-/-}$ mice with 4T1 tumor, lysed with Rapigest, trypsin digested and analyzed by LC-MS/MS. Relative protein abundances and identifications obtained from LC-MS/MS analysis were utilized as input lists for pathway analysis. 4T1-induced MDSC from BALB/c mice served as control

samples; 4T1/IL-1β-induced MDSC from BALB/c mice and 4T1-induced MDSC from TLR4$^{-/-}$ mice were experimental samples.

Fig 3. MS pathway analysis revealed up-regulation of numerous proteins and pathways in inflammatory MDSC including the Fas pathway and caspase network. *A,* Pathway analysis comparison for 4T1-induced and 4T1/IL-1β-induced MDSC from BALB/c mice. Pathway analysis was performed using Pathway Search Engine in combination with ExPlain TM software. Proteins associated with the caspase network and Fas pathway were up-regulated in inflammatory (4T1/IL-1β-induced) MDSC relative to conventional (4T1-induced) MDSC. *B,* Pathway analysis comparison for 4T1-induced MDSC from BALB/c mice vs. TLR4$^{-/-}$ mice. Proteins were identified and compared as in panel A. *C*, Fas pathway proteins common to MDSC populations. Data are from pooled MDSC from individual BALB/c or TLR4$^{-/-}$ mice with either 4T1 or 4T1/IL-1β tumors.

Fig 4. Inflammatory MDSC from BALB/c mice have increased resistance to Fas-mediated apoptosis. *A,* Inflammatory (4T1/IL-1β-induced) MDSC contain less activated caspase-3 as compared to conventional (4T1-induced) MDSC. Blood leukocytes from BALB/c mice with 4T1 or 4T1/IL-1β tumor were cultured overnight with the Fas agonist mAb Jo2 or a control mAb, and subsequently stained with fluorescent mAbs to Gr1, CD11b, and activated caspase-3. Gr1^{+}CD11b^{+} cells were gated and analyzed for activated caspase-3 by flow cytometry. Control and Jo2-treated samples are shown as blue and red histograms, respectively. The corresponding blue and red bars and their associated numbers indicate the MCF and percent positive cells for each sample. The first peak of each color represents activated caspase 3^{-} cells; the second

peak represents activated caspase 3^+ cells. $p<0.03$ for Jo2-treated 4T1-induced vs. 4T1/IL-1β-induced MDSC. Data are from one of five independent experiments with MDSC from five individual mice. *B,* Inflammatory (4T1/IL-1β-induced) MDSC contain less activated caspase 8 as compared to conventional (4T1-induced) MDSC. Blood leukocytes from BALB/c mice induced by 4T1 or 4T1/IL-1β tumor cells were treated as in panel A, except they were stained for activated caspase 8. Data are from one of three independent experiments with three individual mice. *C,* Inflammatory MDSC contain less activated caspase 3 than conventional MDSC. Lysates of Jo2-treated conventional and inflammatory MDSC were electrophoresced by SDS-PAGE and western blotted with mAbs to activated caspase 3. Bands were quantified by densitometry. Normalized density of conventional and inflammatory MDSC treated with Jo2 were 1.31 and 0.74, respectively. Data are from one of two independent experiments with MDSC pooled from 1-2 mice per group. *D,* Conventional and inflammatory MDSC express similar levels of Fas. Blood leukocytes from BALB/c mice with 4T1 (top panel) or 4T1/IL-1β (bottom panel) tumors were stained with fluorescent mAbs to Gr1, CD11b, and Fas. $Gr1^+CD11b^+$ cells were gated and analyzed for cell surface expression of Fas by flow cytometry. Bars indicate the percent Fas^+ cells and the MCF of the Fas^+ cells. *E,* Inflammatory MDSC are more viable than conventional MDSC following treatment with a Fas agonist. Blood leukocytes from BALB/c mice with 4T1 or 4T1/IL-1β tumors were cultured overnight with the Fas agonist mAb Jo2 and assessed the next day for viability by trypan blue dye exclusion. Leukocytes were >90% $Gr1^+CD11b^+$. Data are from one of five independent experiments with MDSC from individual mice.

Fig 5. Inflammatory MDSC are less apoptotic than conventional MDSC as visualized by confocal microscopy. Blood leukocytes from BALB/c mice with 4T1 or 4T1/IL-1β tumor were treated overnight with the Fas agonist Jo2 mAb or control mAb, and surface stained for Gr1 and CD11b and internally stained with DAPI and mAbs to activated caspase-3. Images are single slices.

Figure 1

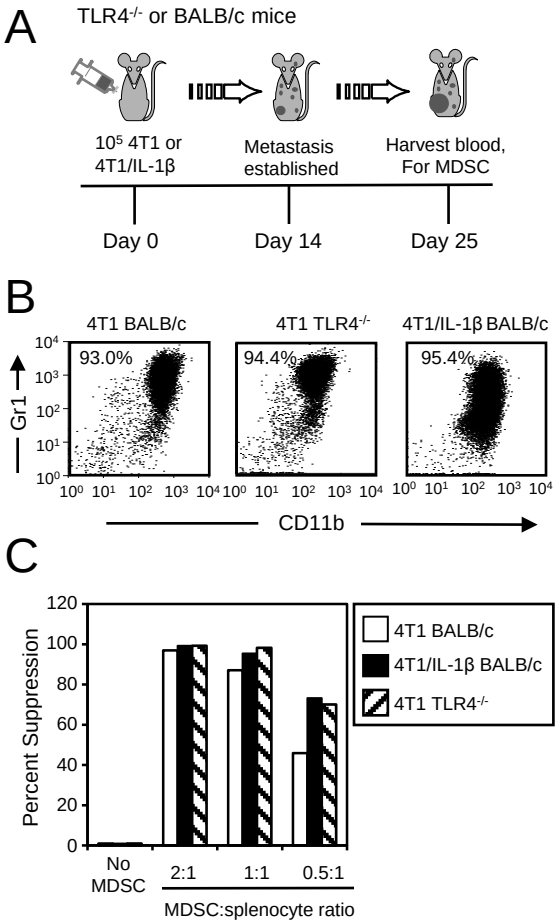

A
TLR4-/- or BALB/c mice
10^5 4T1 or 4T1/IL-1β
Metastasis established
Harvest blood, For MDSC
Day 0
Day 14
Day 25
B
4T1 BALB/c
4T1 TLR4-/-
4T1/IL-1β BALB/c
93.0%
94.4%
95.4%
Gr1
CD11b
C
Percent Suppression
4T1 BALB/c
4T1/IL-1β BALB/c
4T1 TLR4-/-
No MDSC
2:1
1:1
0.5:1
MDSC:splenocyte ratio

Figure 2

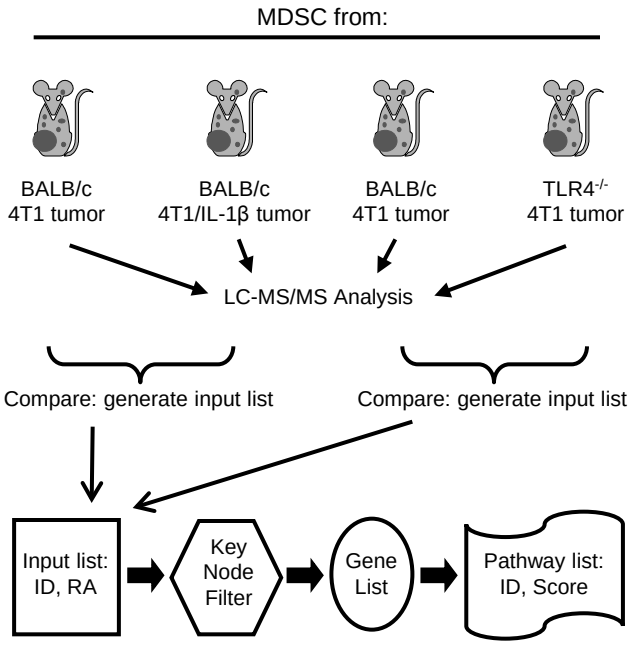
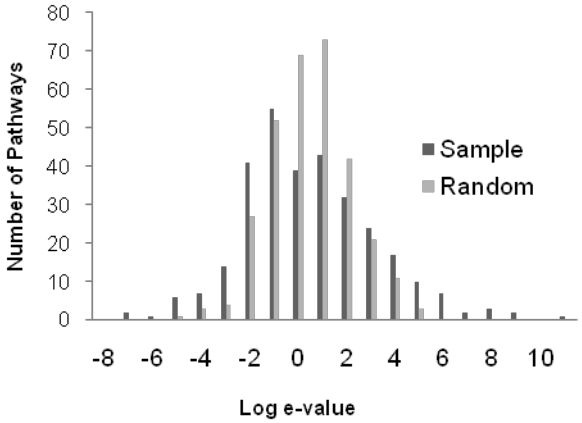

MDSC from:
BALB/c
4T1 tumor
BALB/c
4T1/IL-1β tumor
BALB/c
4T1 tumor
TLR4-/-
4T1 tumor
LC-MS/MS Analysis
Compare: generate input list
Compare: generate input list
Input list:
ID, RA
Key
Node
Filter
Gene
List
Pathway list:
ID, Score

Figure 3

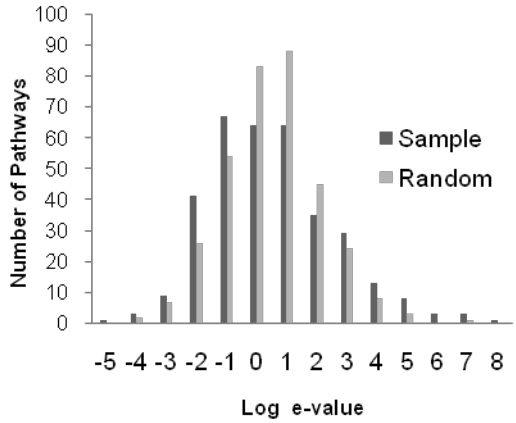
A Comparison of 4T1-induced vs. 4T1/IL-1β-induced MDSC from BALB/c mice



Significantly up-regulated pathways	Log e-value	p-value
Caspase network	10.02	2.28E-11
stress-associated pathways	8.81	4.1E-09
Fas pathway	8.10	6.43E-08
p53 pathway	7.24	1.36E-06
JNK pathway	7.22	1.44E-06
Apo2L pathway	7.22	1.44E-06
p38 pathway	6.29	2.72E-05
TGFbeta pathway	6.24	3.15E-05
B-cellantigenreceptor pathway	5.76	0.000122
RANKL pathway	5.71	0.000139
parkinassociated pathways	5.71	0.00014
TLR3 pathway	5.48	0.000255
IL-1 pathway	5.47	0.000266
TNF-alpha pathway	5.35	0.00036

Significantly down-regulated pathways	Log e-value	p-value
LAT PKC theta	-5.18	0.000543
Sphase(Cdk2)	-5.40	0.000319
G1phase(Cdk6)	-5.61	0.000185
MHC class II NF-AT	-5.63	0.000175
G1phase (Cdk2)	-5.91	8.16E-05
IL-8 pathway	-5.99	6.44E-05
G1 phase (Cdk4)	-6.33	2.41E-05
neurotensin pathway	-7.43	7.21E-07
E2F network	-7.88	1.46E-07

B Comparison of 4T1-induced MDSC from BALB/c vs. TLR4$^{-/-}$ mice



Significantly up-regulated pathways	Log e-value	p-value
insulin pathway	7.12	2.07E-06
T-cellantigenreceptor pathway	6.72	7.46E-06
Apo2L pathway	6.48	1.55E-05
Caspase network	6.03	5.76E-05
tuberin pathway	5.47	0.000266

Significantly down-regulated pathways	Log e-value	p-value
MEKK4 MKK6 ATF-2	-4.88	0.001122
Rac1 p38	-5.02	0.000802

C

Proteins common to MDSC samples

Protein	NCBI Accession number	4T1 BALB/c MOWSE	4T1 BALB/c peptides per protein	4T1/IL-1β BALB/c MOWSE	4T1/IL-1β peptides per protein	4T1 TLR4$^{-/-}$ MOWSE	4T1 TLR4$^{-/-}$ peptides per protein
Caspase 3	gi|6753284	44	2	34	2	56	2
Caspase 8	gi|6753288	37	2	44	2	ND[1]	ND
Lamin B2	gi|52867	425	10	194	8	412	10
Rho GDI beta	gi|13435747	165	4	262	6	387	12

[1] Not detected

Figure 4

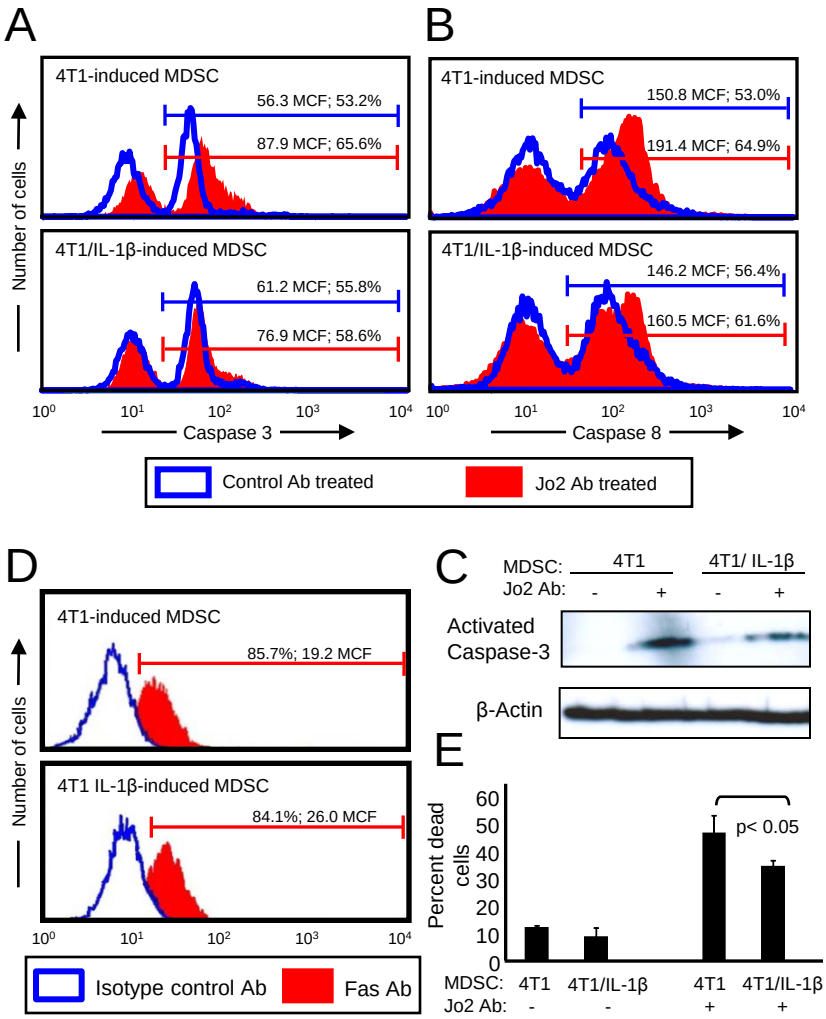
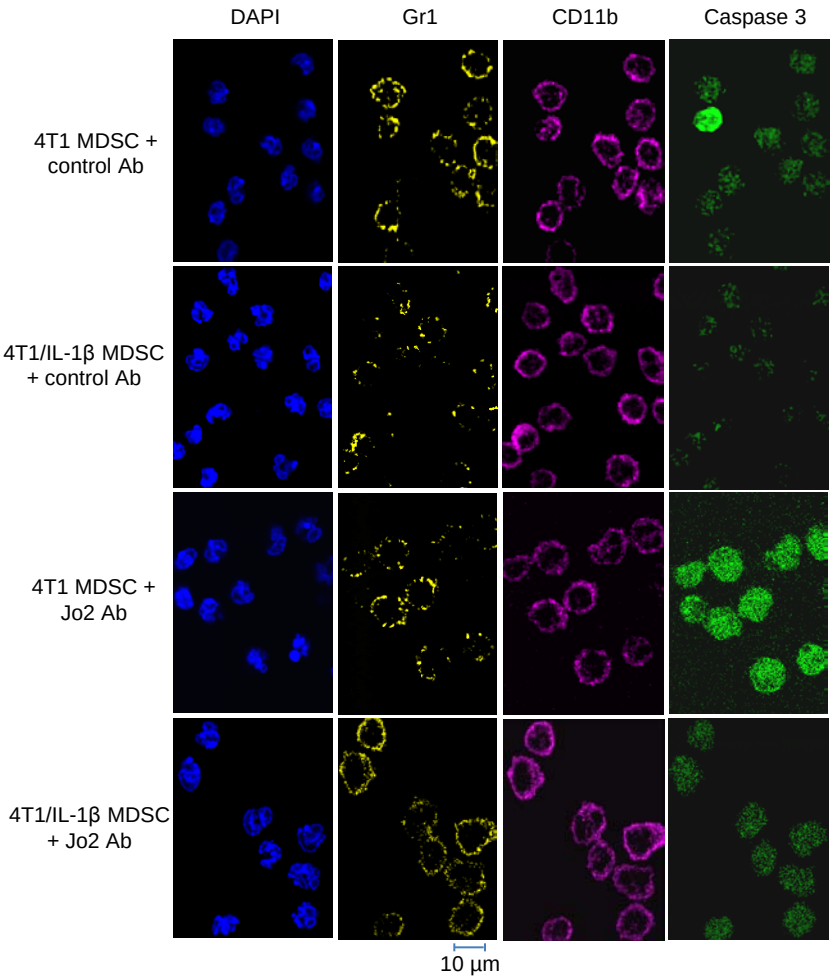

A
4T1-induced MDSC
56.3 MCF; 53.2%
87.9 MCF; 65.6%
4T1/IL-1β-induced MDSC
61.2 MCF; 55.8%
76.9 MCF; 58.6%
Number of cells
Caspase 3
B
4T1-induced MDSC
150.8 MCF; 53.0%
191.4 MCF; 64.9%
4T1/IL-1β-induced MDSC
146.2 MCF; 56.4%
160.5 MCF; 61.6%
Caspase 8
Control Ab treated
Jo2 Ab treated
D
4T1-induced MDSC
85.7%; 19.2 MCF
4T1 IL-1β-induced MDSC
84.1%; 26.0 MCF
Number of cells
Isotype control Ab
Fas Ab
C
MDSC: 4T1 4T1/ IL-1β
Jo2 Ab: - + - +
Activated Caspase-3
β-Actin
E
Percent dead cells
p< 0.05
MDSC: 4T1 4T1/IL-1β 4T1 4T1/IL-1β
Jo2 Ab: - - + +

Figure 5


DAPI
Gr1
CD11b
Caspase 3
4T1 MDSC + control Ab
4T1/IL-1β MDSC + control Ab
4T1 MDSC + Jo2 Ab
4T1/IL-1β MDSC + Jo2 Ab
10 µm